The Hydrolysis of Collagen by Trypsin

STANFORD UNIVERSITY LIBRARIES

DISSERTATION

Submitted in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Pure Science
in Columbia University.

By

FRANK LESLIE SEYMOUR-JONES,
M.C., B.Sc., M.Sc., A.M.

STANFORD UNIVERSITY LIBRARIES
MENDRAWN

NEW YORK CITY
1923

3781
C72S
UNCLASSED
Dissertation

The Hydrolysis of Collagen by Trypsin

DISSERTATION

Submitted in Partial Fulfilment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Pure Science
in Columbia University.

By

FRANK LESLIE SEYMOUR-JONES,
M.C., B.Sc., M.Sc., A.M.

NEW YORK CITY
1923

DEDICATED

TO THE

MEMORY

OF

RICHARD ARNOLD SEYMOUR-JONES,
B.Sc., M.Sc.,

Lieutenant, 4th Battalion, South Lancashire Regiment.
Killed in Action, Flanders, 27th March, 1915

"Tros ryddid gollasant eu gwaed."

ACKNOWLEDGMENT

This investigation was carried out under the direction of Professor Arthur W. Thomas. To him my thanks are due for his unremitting interest and ever-ready advice.

THE HYDROLYSIS OF COLLAGEN BY TRYPSIN

Historical Introduction

It is commonly stated in the literature of proteins[1] and of leather chemistry[2] that collagen is not subject to tryptic hydrolysis unless it has been previously swollen with acids or alkalies, shrunk in water at 70°, or treated with pepsin. Plimmer[3] advanced the theory that "those proteins which are resistant to the action of trypsin until they have been acted upon by pepsin will have all their units contained in the anhydride ring," and this has been used by Wilson[2] in postulating a ring structure for collagen. In a private communication, received in reply to one describing preliminary findings that collagen was hydrolyzed by trypsin, Plimmer states that "as far as I know there is no direct evidence that collagen has a ring structure. The statement is one of supposition. There is also no evidence that proteins have a ring structure; if they consist of a chain of amino acids in polypeptide combination, there is no means of introducing a ring, except just to join the two ends. There is no doubt that trypsin acts upon gelatin and probably also on collagen. Some change evidently occurs in the formation of gelatin from collagen, but its nature is unknown. Many of the older workers only used trypsin as hydrolyzing agent for a short period of time and the old statement is probably more one of comparison than of actual facts. They did not see collagen dissolve, therefore it was not acted upon."

A review of the literature reveals the fact that all statements as to the non-hydrolyzing action of trypsin on collagen date back to work by Kühne[4], Ewald and Kühne[5], and Ewald[6], in 1887 and 1890. Kühne was the first to isolate trypsin and to study its action on vital tissues. This work was based on visual, qualitative observation only, for very limited periods of time, on tissues as found (i. e., in bulk, not ground up), and without any consideration of the significance of the reaction of the solution in contact with the substrate.

Object of Investigation

Preliminary experiments demonstrated that trypsin had a hydrolytic action on collagen. Since collagen is insoluble in water, the reaction appeared of unusual interest, in that an enzyme would be acting on an insoluble, though hydrated substrate. The difference is probably largely one of degree, for we may equally well say that water is soluble in collagen. It was furthermore desirable to clear up the very indefinite statements of the effect of previous treatment with acids and alkalies. Yet further, since collagen combines with various substances to form leather, a process the theory of which is still almost entirely speculative, it seemed possible that interesting

results might be obtained by subjecting collagen, tanned in various ways, to the action of trypsin. Not only would this help in elucidating the theory of tanning, but it might also give some idea of the mechanism of tryptic hydrolysis.

It was therefore decided to investigate the action of trypsin on collagen quantitatively, with special reference to the control of acidity, both in the pre-treatment of the collagen and during contact of the enzyme with the collagen. The object was to determine the effect, if any, of pre-treatment at different hydrogen-ion concentrations and to obtain more exact data of the optimum conditions for the action of trypsin on collagen. These data having been obtained, they could then be applied in a study of the action of trypsin on collagen tanned in different ways.

Materials Used

Since the substrate to be used was solid and insoluble, to get a maximum effect it was essential to expose as great a surface as possible to the action of the enzyme. Hence "Standard Hide Powder, 1921,"* was chosen as the source of collagen, both as the most convenient, pure form for quantitative work and in view of its importance in leather chemistry, where it is widely used in analysis and research. This is prepared by grinding up into powder hides which have been unhaired by liming and subsequently delimed by treatment with hydrochloric acid. Previous to use in this investigation it was rendered fat free by extraction with chloroform and was sifted through sieves of known mesh so as to obtain a uniform product. It contained collagen, a little elastin, and traces of keratins from the cell walls, with possibly a small amount of early degradation products of incipient hydrolysis. The amount of these latter in Standard Hide Powder is such that 6.5 g. of the dry hide powder shaken with 100 c.c. water yields less than 5 mg. of soluble organic matter.

Two samples of trypsin were used, "Pancreatin" of the Parke, Davis Co. in the preliminary work, and "Trypsin" of Fairchild Bros. and Foster of New York City, throughout the whole of the quantitative measurements.

This "Trypsin" was tested on a casein substrate, according to the conditions laid down by Sherman and Neun (*J. Am. Chem. Soc.*, 38, 2199 (1916)), namely, 2 mg. of trypsin acting on a casein substrate at $-\log C_H{}^+ = 8$ at 40.00° for half an hour. Under these conditions 2 mg. of the enzyme gave 17.7 mg. of soluble nitrogen. This represents a trypsin strength approximately one-third that of the strongest, high grade commercial preparation used by Sherman and Neun. As much as 20 mg. of the enzyme tested under the conditions requisite for pepsin activity gave no soluble nitrogen whatever.

* Manufactured by the Standard Mfg. Co., Ridgway, Pa.

The acidity was controlled by a series of buffer solutions, the hydrogen-ion concentration being determined electrometrically. The buffer mixtures covered a range of $-\log C_{H^+} = 1.9$ to 9.9, and consisted of acetic acid-sodium acetate, sodium citrate, potassium and sodium phosphates, and sodium borate, with hydrochloric acid or sodium hydroxide added as required.

The other chemicals used were the standard, laboratory "chemically pure" products.

General Method

About 0.5 g. of hide powder was placed in a 10 c.c. centrifuge tube with a conical bottom, graduated in tenths of a cubic centimetre. Five or ten c.c. of a buffer solution were added, together with one or two drops of toluene. The tubes were then corked and fastened to a shake machine, rotating at 8 r.p.m. After rotation for a definite time, the tubes were removed and centrifuged for twenty minutes at 1200 "times gravity." The supernatant liquor was poured off and five or ten c.c. of fresh buffer solution added of the same hydrogen-ion concentration as that to be employed in the digestion. The hide powder was stirred up in this, one or two drops of toluene added, and the shaking repeated to bring the hide powder to the new hydrogen-ion concentration. The tubes were then centrifuged as before and the volume of hide powder noted.

The supernatant liquid was poured off from the tubes, five or ten c.c. of trypsin solution, made up in a buffer solution, added, together with toluene as before, and the shaking repeated. This shaking was carried out in a Freas water thermostat maintained at 40.00°. The tubes were finally centrifuged for twenty minutes at the same speed, the volume of hide powder remaining undissolved noted, and hence the amount digested by the action of the trypsin determined.

A series of sixteen centrifuge tubes was employed, all experiments being performed in duplicate or more, and satisfactory checks being obtained. Control tubes were run in every case, under identical conditions, except that the trypsin was omitted. The pre-treatment was carried out at first at 40.00°, but subsequently at room temperature in order to minimize hydrolysis. The tryptic digestions and their controls were always carried out at 40.00°.

The accuracy obtained in this method of measurement, which was adopted throughout the whole series of experiments, is limited entirely by that in reading the level in the tubes. With the centrifuge and the fine sifted hide powder it was possible to obtain a well defined boundary, and the percentage digestions so obtained are accurate to ± 2.

One possible objection to this method is the heat effect on swelling. It is known that increased temperature decreases the swelling of hide powder, and hence hide powder measured first after treatment

at room temperature (20-30°) and then after treatment at 40° might be expected to show differences in volume. But the speed of revolution of the centrifuge raises the temperature of solutions placed therein to 30-35°, and in practice it was found that any error from this direction fell well within the limits of error of the method, as is shown in the numerous zero controls found in the later stages of the work with quinone tanned hide powder.

Considering the insoluble nature of the substrate, the method is probably the most accurate available, whilst being reasonably rapid. It is distinctly preferable to filtering off the undigested hide powder and determining that dissolved by an estimation of nitrogen in the filtrate. This latter is objectionable and inaccurate for several reasons. Small quantities of liquid only are available. It is difficult to filter off the undigested hide powder satisfactorily and to obtain a clear filtrate; the degradation products of hydrolysis are partially molecularly and partially colloidally dispersed, and any filtration will merely effect an arbitrary separation, dependent on the size of the filter pores. More particularly, part of the nitrogenous matter in solution will be absorbed by the undigested hide powder, and a true aliquot will be impossible.

Preliminary trials indicated that the hide powder was very considerably hydrolyzed by trypsin at 40.00°, though not greatly attacked at room temperature.

Effect of Pre-Treatment

The hide powder was shaken in buffer solutions of $-\log C_H{}^+ =$ 1.1 to 8.9 for three hours, then for a further three hours in buffer solutions of $-\log C_H{}^+ = 5.3$ to 7.9, and finally subjected to the action of a 0.5 per cent trypsin solution at the same hydrogen-ion concentration for six hours at 40.00°. The results are summarized in Table I.

TABLE 1.

Pre-treatment at $-\log C_H{}^+$	Digestion at $-\log C_H{}^+$	*Per cent hydrolysis:* Trypsin	Control
3.0)	5.3	(69	7
5.3)		(65	8
3.0)	5.9	(81	20
5.3)		(89	34
7.0)		(88	42
8.9)		(87	46
1.1*)	7.0	(73	53
3.0)		(73	6
5.3)		(75	18
7.0)		(80	32
3.0)	7.9	(73	25
5.3)		(80	54
7.0)		(81	72
8.9)		(80	69

* Approximately 0.1 *N*. HCl used.

It is clear from the above that the pre-treatment at different acidities exerts no appreciable influence on the subsequent hydrolysis of the collagen by trypsin. It appears, however, to increase the ordinary hydrolysis at extreme acidity and with increasing alkalinity of pre-treatment.

Optimum Hydrogen-Ion Concentration for Tryptic Hydrolysis

It was evident from the foregoing that tryptic hydrolysis of collagen was greatest in the region around $-\log C_H{}^+ = 5.9$. Hence a series of experiments was made between $-\log C_H{}^+ = 5.3$ and 7.0, varying the hydrogen-ion concentration by 0.2—$\log C_H{}^+$ each time. The hide powder was shaken for three hours in a buffer solution at the desired hydrogen-ion concentration, then digested at the same concentration for 15 minutes with a 0.05 per cent trypsin solution. The results are shown in Table 2.

TABLE 2.

	Per cent hydrolysis	
$-\log C_H{}^+$	Trypsin	Control
5.3	69	21
5.5	70	10
5.7	71	13
5.9	72	14
6.1	73	15
6.3	69	19
6.5	66	17
6.7	69	12
7.0	70	18

The optimum range extends from 5.7 to 6.1 $-\log C_H{}^+$, the logarithmic mean of which is 5.87. Consequently the optimum hydrogen-ion concentration for the reaction may be taken as $-\log C_H{}^+ = 5.9$.

Time Effect and Velocity Constant

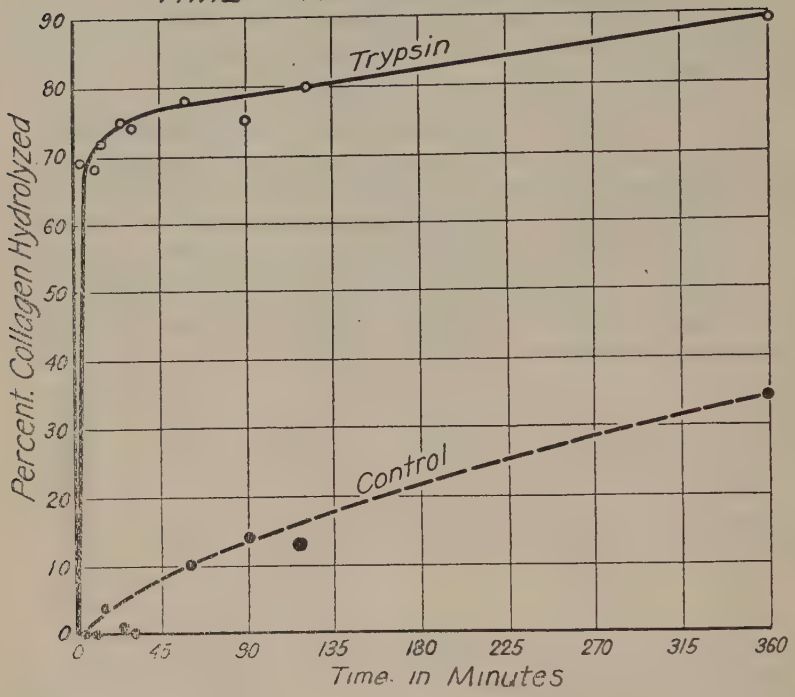
The hide powder was shaken for three hours in a buffer solution of $-\log C_H{}^+ = 5.9$, centrifuged and measured, then digested with 0.5 per cent trypsin solution of the same reaction at 40.00° for times from five minutes to six hours. Immediately on removal from the thermostat the tubes were centrifuged and measured. The results are given in Table 3 and Figure 1.

TABLE 3.

Time	*Per cent hydrolysis*		
(minutes)	Trypsin	Control	k
5	69	0	0.102
10	68	0	0.050
15	72	4	0.037
20	75	1	0.030
30	74	0	0.020
60	78	10	0.011
90	75	14	0.007
120	80	13	0.006
360	89	34	0.003

FIG. 1

TIME - HYDROLYSIS CURVE



Owing to the time taken for the placing of trypsin solution in the tubes, for the tubes and solutions to reach 40° in the thermostat, and for the transfer of the tubes to the centrifuge, the times given are more comparative than exact.

The curve so obtained is similar to that commonly found for the action of trypsin on proteins in solution. From the data the velocity constant, k, was obtained by means of the uni-molecular reaction formula, using natural logarithms, and is inserted in the table. This "constant" steadily decreases with time, very much in the same ratio as in a case cited by Bayliss[7] for the action of trypsin. This may most probably be accounted for by (a) the disappearance or destruction of the enzyme, (b) the effect of the hydrolysis products in reversing the reaction or destroying the enzyme, (c) removal of the enzyme by its combination with the substrate or with hydrolysis products.

Concentration of Enzyme

The concentrations of trypsin employed generally in the foregoing experiments were high, in order to obtain a definite and well marked result. In the next set of experiments the concentration of enzyme

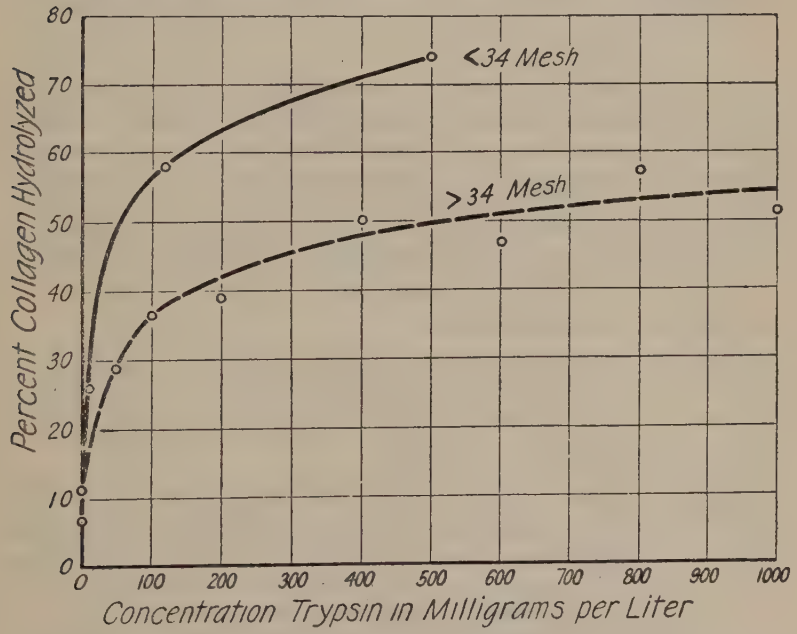
was varied. The procedure was as before, digestion being carried out at $-\log C_H{}^+ = 5.9$ and 40.00° for thirty minutes. The results are given in Table 4 and Figure 2.

TABLE 4.

Concentration of trypsin mg. per liter	Per cent hydrolysis	
492.00	74	
123.00	58	
12.30	26	
6.15	6	) Figures value-
3.00	5	) less owing to
1.50	11	) size of con-
0.30	12	) trol.
0.15	10	)
0.00 control	7	

FIG. 2

CONCENTRATION OF TRYPSIN AND SIZE OF PARTICLES



There appears to be a limiting value at a trypsin concentration of about 10 mg. per liter, below which the ordinary hydrolysis is equal to that engendered by the enzyme.

A measurement of the hydrogen-ion concentration of the residual liquor from the above after hydrolysis gave—$\log C_H{}^+ = 5.8$,

showing that it had not been appreciably changed by the reaction products of the hydrolysis.

Size of Substrate Particles

The hide powder employed in the preceding experiments consisted of that portion of "1921 Standard Hide Powder" which passed through a sieve of 34 meshes to the inch. The coarser particles retained by the sieve were next used. Hydrolysis was carried out as before, using 0.5 g. of hide powder in a buffer solution of $-\log C_H{}^+ = 5.9$, with varying concentrations of trypsin, for 30 minutes at 40.00°. The results are given in Table 5 and Figure 2.

TABLE 5.

Concentration of trypsin mg. per liter	Per cent hydrolysis
1000.0	51
800.0	57
600.0	47
400.0	50
200.0	39
100.0	36.5
50.0	29
0.0 Control	11.5

The larger and less uniform size of the hide powder particles, not only making an even level in the centrifuge tube difficult to obtain but also offering a varying surface for the enzyme action, make the results somewhat irregular. The lessened total surface and the greatly lessened specific surface of the substrate particles naturally lead to a lower rate of hydrolysis. The limiting concentration of enzyme appears to be of the same order of magnitude as with the fine hide powder.

Completeness of the Hydrolysis

In none of the foregoing had one hundred per cent hydrolysis been obtained with the trypsin. An attempt was therefore made to bring the hydrolysis to completion by removing the reaction products from contact with the substrate.

Some 0.5 g. of fine hide powder was placed in a series of tubes and shaken with 5cc. of a phosphate buffer solution ($-\log C_H{}^+ = 5.9$), as before, for three hours at room temperature. The tubes were then centrifuged and the volume of hide powder measured. The supernatant liquor was poured off. To half the tubes 5cc. of a 0.1 per cent trypsin solution (made up in the same buffer) were added, and to the other half 5cc. of the same buffer. These were shaken for twenty minutes in the thermostat at 40.00°, centrifuged, and measured. The liquid was poured off, fresh trypsin and buffer solutions added, and the process repeated, four times in all. The experiment was stopped after eighty minutes owing to the increasing hydrolysis in the control. The results are shown in Table 6 and Figure 3.

FIG. 3

RESIDUE DIGESTION

RENEWING TRYPSIN EACH 20 MINUTES

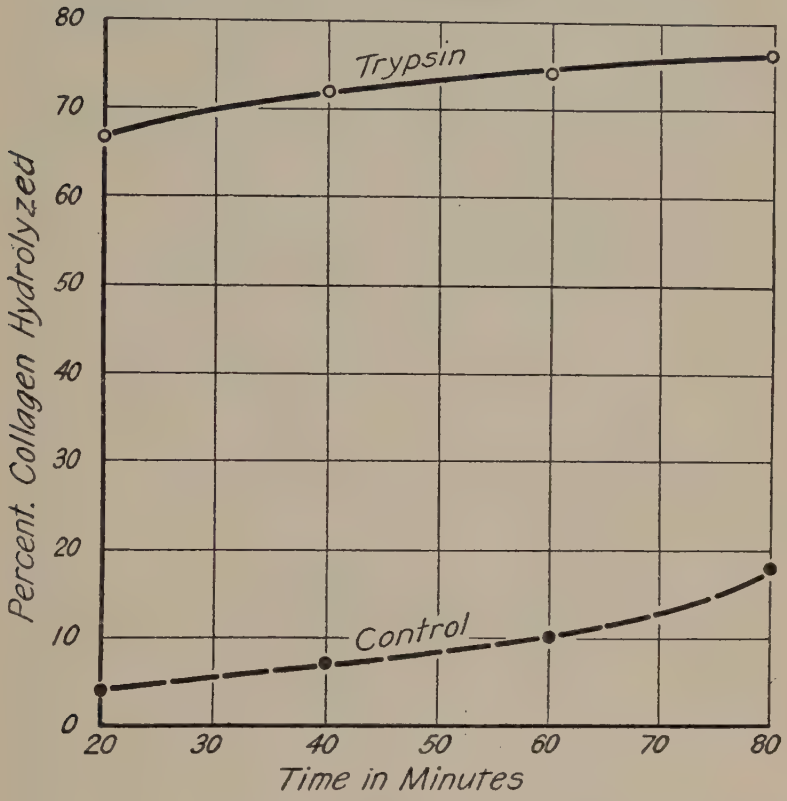


TABLE 6.

Time	*Per cent hydrolysis* Trypsin	Control
First 20 minutes	67	4
Second 20 minutes	72	7.5
Third 20 minutes	74	10
Fourth 20 minutes	76	18.5

The curve of tryptic hydrolysis is slightly concave to the time axis, and hence it is probable that of itself it never proceeds to completion. It is not possible to test this with hide powder alone, since increasing the time greatly increases the hydrolysis in the control.

This result agrees with the work of Anderson[8], who found it to be practically impossible to obtain complete hydrolysis of proteins by enzymes *in vitro*, although Abderhalden and Rona[9] claimed to have

obtained complete hydrolysis of meat protein by digestion for three to four weeks at 37° with pepsin, trypsin and erepsin.

Reversibility of the Reaction

The reaction under consideration is one of action on an insoluble substrate and hence it is obvious that hydrolysis due to the trypsin can only occur when the trypsin is in contact with the hide powder. The hide powder is swollen and hydrated, and any diffusion of trypsin into the interior of the particles is slow. This is shown by the difference between the effects with fine and coarse hide powder and the slowness of the effect on skin. The action of trypsin under the conditions of the experiments may then be taken to occur almost entirely at the surface of the hide powder, any action in the interior of the particles being negligible in such short spaces of time. That trypsin does actually slowly diffuse into the skin is demonstrated in a process called bating in leather manufacture, which is discussed later on.

If, in Figure 1, in place of calling the ordinate "per cent collagen hydrolysed," it be called "concentration of collagen" (since all action takes place in the collagen phase) and the figures be reserved (i. e., 100 per cent hydrolysis=0 per cent collagen), we then have the usual reversible equilibrium curve. In view of the surface action this may not be wholly justifiable, but it is here the only means possible. The reverse curve, that of the formation of collagen from hydrolysis products, it is of course not possible to obtain. Yet the curve shows that there is a perceptible, although a very slight reversibility, as would be expected, since the curve approaches but does not touch the zero per cent collagen line. The question is further complicated by the fact that we have, as with all proteins, not a simple hydrolysis, but a whole series, from collagen through gelatin and intermediate stages down to amino-acids. Here the hydrolysis of collagen to gelatin is so slow compared with the hydrolysis of gelatin by trypsin that it is substantially the kinetics of the former reaction which are represented in the curve, but it must nevertheless be borne in mind that the trypsin curve represents the whole series of actions, while the control curve merely covers the change from collagen to gelatin.

The general resemblance of the curve of trypsin with hide powder (Figure 1) to those obtained with trypsin and soluble proteins—soluble in so far that they are probably colloidally dispersed, or the protein ion exists as an ionic micelle or aggregate—leads one to suspect that, since with trypsin-collagen the main action is undoubtedly at the surface, the action with trypsin-soluble protein is probably also at the surface of the colloidally dispersed protein. It must also be remembered that the trypsin itself is in all likelihood also colloidally dispersed.

Practical Applications

In leather manufacture certain skins are treated in trypsin solutions, whereby the elastic fibers (elastin) are removed from the

pelt, leaving the collagen fibers apparently unattacked, at least as far as can be ascertained by staining and microscopic observation. This process is known as *puering* or *bating*. The skin at the same time "falls"; i. e., becomes flaccid, this action being due chiefly to the acidity of the liquor. Superficially, unless bating be unduly prolonged, the difference between a bated and an unbated skin is merely one of feel and extensibility.

Wood[10] states that "a fallen (i. e., bated) skin contains less water than when in the swollen condition, but the difference between the percentages of water in the skin in the two states is relatively small." According to him, "from 3 to 8 per cent of water is expelled during puering." A bated (puered) skin contains from 78 to 80 per cent of water. Rosenthal[11] estimates the elastin content of limed and washed calfskin at from 5 to 9 per cent. This is, however, by tryptic digestion, and the figure is certainly excessive, some collagen being included therein. Probably the actual content of elastin does not exceed 3 per cent, as experiments on the hydrolysis of hide powder with dilute acids (which attack the collagen but not the elastin at boiling temperature) indicate.

To examine this apparent discrepancy of the non-digestion of collagen in practice with the ready digestibility found in these experiments, some experiments were made with guinea pig skins. A skin was soaked in water, unhaired in a sodium sulphide-lime liquor, the flesh thoroughly removed, and the skin then completely delimed in dilute acetic acid. It was well washed, then drummed in several changes of a phosphate buffer solution ($-\log C_H{}^+ = 5.9$) for six hours. It was trimmed and four strips, about 6 inches by 1 inch, cut out. The strips were blotted in a clean cloth to remove surface moisture and weighed. Two strips were then treated for periods of three and seven hours successively in trypsin solutions of 0.1 and 0.4 per cent at 40.00°, the other two strips receiving identical treatment but without trypsin. They were then blotted off and weighed as before.

The trypsin treated strips showed a percentage loss in weight of 38 after three hours and 48 after ten hours, while the controls gave 18 and 25 per cent loss respectively. Even allowing for loss of elastin and water, these very approximate results show a very definite digestion of collagen by the trypsin. Further, the trypsin treated skins were thin and could be stretched out to 10 inches without springing back, i. e., they exhibited good "stand." The controls were somewhat "down" (i. e., flaccid), but were still firm and inextensible.

Upon communication of these results to Mr. J. A. Wilson, he examined the effect on calfskin, and found[12] that, after a preliminary passive period of about 24 hours, it could be almost completely hydrolyzed by trypsin.

That none, or practically none, of the collagen is removed in commercial bating is due to the fact that no such concentrations of

trypsin as were used in these experiments are ever likely to be present in ordinary bate liquors. Yet it is quite possible, with careless supervision, to get "over-bating" and disintegration of the skins in practice, possibly due in part to bacterial action. It must also be remembered that the relatively large size of the collagen fibrils and the organized skin structure not only hinder diffusion of the trypsin into the skin, but also retard diffusion of the reaction products, which will accumulate on the surface of the collagen fibers and so protect them from further tryptic action.

Effect of "Charge" on Trypsin

Trypsin, in common with several other enzymes, is supposedly amphoteric. Michaelis and Davidsohn[13], using the electrophoresis and flocculation (minimum solubility) methods, found the value 1.35—$2.6 \times {}_{10}{}^{-4}$ *N.* hydrogen ion for the isoelectric point of this protease.

All work hitherto was carried out at such hydrogen-ion concentrations that the substrate and enzyme were both "negatively charged," i. e., anions. It seemed of interest to examine the hydrolysis which would occur when the hydrogen-ion concentration was so controlled that (a) the substrate and enzyme would be "oppositely charged," the collagen being "positive" (cationic) and the trypsin "negative" (anionic), (b) both substrate and enzyme would be "positively charged."

An attempt was made to determine the iso-electric point of the sample of trypsin used, without any special purification of the enzyme. The U-tube electrophoresis technique was employed, adding maltose to raise the specific gravity of the trypsin solution and employing an albumen solution in the arms above the trypsin solution to obviate surface tension effects. Despite all precautions, in every case, even so far away from the supposed iso-electric point as — log $C_H{}^+$=5, migration of trypsin to both anode and cathode occurred.

Nevertheless hydrolysis sets with 0.5 per cent trypsin solution were run at — log $C_H{}^+$ = 3 and 4.5, using 100-mesh hide powder, digestion being for twenty minutes at 40.00°. The results are given in Table 7.

TABLE 7.

Digestion at — log$C_H{}^+$	*Per cent hydrolysis*	
	Trypsin	Control
3.0	27	2
4.5	82	6

Even if the trypsin and collagen be oppositely charged at — log $C_H{}^+$ = 4.5, their mutual precipitation appears not to affect the hydrolysis. However, it would appear extremely doubtful whether the trypsin actually has a true iso-electric point. While in the

electro phoresis experiments at $-\log C_H{}^+ = 5.0$ most of the trypsin was found to migrate to the anode, yet some trypsin undoubtedly migrated to the cathode. That the hydrolysis is so slight at $-\log C_H{}^+ = 3.0$ must be attributed to the destructive effect of such acidity on the enzyme.

The Action of Trypsin on Tanned Collagen

The primary objection to the hydrolysis by trypsin of collagen which has been treated with various tanning agents lies in the fact that most of the substances used for tanning, e. g., heavy metal salts and formaldehyde, are very definite enzyme poisons. Nevertheless it seemed at least possible that by thorough washing of the treated hide powder all soluble and ionized matter could be removed, leaving merely the insoluble combination of collagen and tanning agent. There then seemed no reason why a more complex compound, such as that of the collagen-tanning agent, should not be hydrolyzed by trypsin, just as collagen alone is hydrolyzed. It has, however, to be remembered that these various tanned collagen compounds are in general characterized by the fact that, unlike collagen alone, they are not sufficiently attacked by boiling water as to yield gelatin, although some, e. g., vegetable tanned collagen, are not entirely unchanged by hot water. There is further the possibility that the trypsin might hydrolyse the tanned collagen sufficient to liberate enough of the tanning agent which would inhibit the further action of the trypsin by "poisoning" it, i. e., presumably by combining with or precipitating it.

Yet the possibilities of throwing light on (a) the point of attack of the trypsin in the collagen molecule and (b) the nature of the combination in each tannage, seemed such that they warranted the employment of this distinctly novel method of attack.

The 100-mesh fine siftings of hide powder were therefore tanned in solutions of basic chromium sulphate, quinone, formaldehyde, copper sulphate, and gallotannin, as described in detail below individually. The tanned powders were then dried out in an oven at 38-40°, powdered up again, soaked once more in buffer solution at $-\log C_H{}^+ = 5.9$, half gram samples digested with 10cc. of 0.5 per cent trypsin solution made up in the same buffer solution for twenty minutes at 40.00°, and the loss in volume measured by the centrifuge method as before. Blanks were run in every case, omitting the trypsin.

Chrome Tanned Hide Powder

In the first place a series of ten test tubes, each containing 10cc. of gelatin jelly, was set up. On top of these jellies were placed divers solutions, and the results after standing four days at room temperature noted. These are shown in Table 8.

TABLE 8.

Tubes. No.	Supernatant solution.	Observation after 4 days.
1, 2.	2cc. water	No effect.
3, 4.	2cc. 0.5% trypsin plus 0.5cc. dil. $Cr_2(SO_4)_3$	Diffusion of Cr into jelly. Much liquefaction.
5, 6.	2cc. 0.5% trypsin plus 0.5cc. dil. Cr alum	Diffusion of Cr into jelly. Very slight liquefaction on surface.
7, 8.	2cc. 0.5% trypsin	Much liquefaction.
9.	2.5cc. dil. Cr alum	Diffusion of Cr into jelly
10.	2.5cc. $Cr_2(SO_4)_3$	No liquefaction

These experiments indicated that hydrolysis of chrome tanned hide powder might also be expected.

A chrome liquor was made up of approximate composition $Cr(OH)SO_4$, from chromium sulphate and sodium hydroxide, to give 1.04 per cent Cr. Ten g. of 100-mesh hide powder was tanned in 200cc. of this liquor (A) and 10 g. further in 200cc. of the stock liquor diluted 1:4 with water, i. e., 0.21 per cent Cr (B), for 22 hours on the shake machine. The tanned powders were thoroughly washed with distilled water until no test for Cr^{+++} could be obtained, then were dried in the oven at 75°.

Hydrolysis was then carried out as described above, the results being given in Table 9.

TABLE 9.

Chromed Hide Powder	*Percentage hydrolysed*	
	Trypsin	Control
A	3.6	0.0
B	6.9	0.0

Further sets of hide powder were tanned in basic chromium sulphate solutions of various concentrations, but when the tanned powder was subsequently treated at $-\log C_H{}^+ = 5.9$ some of the chromium was stripped from the leather. Even with this, only the lightest tanned powder showed any digestion with trypsin. It was not found possible to prepare chrome liquors for tannage at $-\log C_H{}^+ = 5.9$, since the chromium began to precipitate out at this hydrogen-ion concentration.

It appears from this that where hide powder is completely chrome tanned, it is not hydrolysed by trypsin. Whether this is due to the poisoning action of the chromium on the trypsin, or to the chromium masking the linkage which is attacked by trypsin is not definitely established. In any case the theory of chrome tannage[14] is at present in so uncertain a condition that no light can yet be thrown on the action of trypsin in this particular reaction.

Quinone Tanned Hide Powder

About 10 g. of 100-mesh hide powder was drummed up for 24 hours in each of three solutions of 1.0, 0.5 and 0.25 per cent quinone respectively, at $-\log C_H{}^+ = 5.9$, then allowed to stand for 48 hours, thoroughly washed to remove all uncombined quinone, and dried out at 40°. A further set, using 0.1 per cent quinone, was discarded, as the hide powder was obviously incompletely tanned. These tanned powders were analyzed, and gave the following results:

Powder tanned in quinone solution	Water	Hide Substance (N x 5.62)	Quinone (by difference)
1 per cent	8.61	84.09	7.30
0.5 per cent	8.79	86.70	4.51
0.25 per cent	9.17	89.60	1.23

Half gram lots of these three tanned hide powders together with some untanned hide powder were digested with trypsin as described before. Subsequently the residual liquors were poured off, fresh trypsin and buffer solution added, and a further digestion period of 20 minutes given. The results are given in Table 10.

TABLE 10

Hide powder tanned in quinone solution	*Per cent hydrolysis* 1st 20 minutes		2nd 20 minutes	
	Trypsin	Control	Trypsin	Control
1.0 %	33.5	0	61	0
0.5 %	50	0	69	0
0.25%	68	0	79	0
0.0 (Untreated)	86	13	90.5	23

Considerable quinone and hydroquinone were liberated during hydrolysis.

It is known that this is a true tannage. Neither quinone nor hydroquinone appear to destroy or combine with the trypsin. The group at which the collagen normally hydrolyses with water appears to have been so changed by the tannage that hydrolysis no longer occurs. With the trypsin, some masking of the attacked group is shown by the lessened hydrolysis, which degree of hydrolysis varies with the amount of quinone used in tanning (i. e., in combination with the collagen).

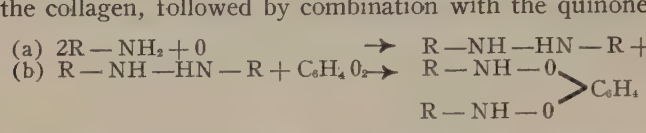
Quinone tannage was discovered by Meunier[15] in 1908. His view[16] of the process is that it takes place by oxidation of the collagen and reduction of the quinone. Representing the collagen as R-NH_2, we may have either

(a) $R-NH_2 + 2C_6H_4O_2 \rightarrow R-N\begin{matrix}O\\O\end{matrix}>C_6H_4 + C_6H_4(OH)_2$

(b) $2R-NH_2 + 3C_6H_4O_2 \rightarrow \begin{matrix}R-N-O\\ |\\ R-N-O\end{matrix}>C_6H_4 + 2C_6H_4(OH)_2$

These reactions are exactly similar to those between quinone and aromatic amines. Support is lent to this view by the fact that hydroquinone is found in the solution after tannage.

Fahrion[17], on the other hand, presumes a preliminary oxidation of the collagen, followed by combination with the quinone.



This view does not explain the presence of hydroquinone in the residual liquor, unless it be supposed that the oxygen necessary for the first reaction is furnished by reduction of the quinone.

Whichever view of the mechanism of the tannage be accepted, it is the amino-group which is linked through oxygen to the benzene ring. The quinone tanned collagen is readily hydrolysable by trypsin, although not quite so rapidly as the untanned hide powder. The combination therefore exerts only a slight retarding effect.

Formaldehyde Tanned Hide Powder

About 10 g. of 100-mesh hide powder was drummed for 24 hours in each of four solutions of (a) 1.90, (b) 0.95, (c) 0.38, (d) 0.19 per cent formaldehyde respectively at $-\log C_H{}^+ = 5.9$, then allowed to stand for 21 days, filtered, thoroughly washed, dried at 40° and powdered. These powders gave on analysis:

Hide Powder	Water %	Hide substance %	Formaldehyde (by difference)
a	7.05	87.84	5.11
b	6.99	88.81	4.20
c	6.36	90.34	3.30
d	8.20	89.02	2.78

Half gram lots of these tanned hide powders were digested with trypsin, as described before. The results are given in Table 11.

TABLE 11.

Hide powder tanned in HCHO solution	*Per cent hydrolysis*	
	Trypsin	Control
1.90 %	6	5
0.95 %	8	14
0.38 %	18	3
0.19 %	35	0
0.00 % Untanned	86	13

The results, particularly in the controls, are somewhat irregular. Formaldehyde was found to be present in all the residual liquors after digestion, and its destructive action on the trypsin may well account for the comparatively small hydrolysis in the more heavily tanned powders.

Tannage with formaldehyde is of the type most generally

acknowledged to be purely chemical in nature. It is usually represented as

$$R\langle^{NH_2}_{COOH} \text{ or } R\langle^{NH_3}_{COO} + H.CHO \rightarrow R\langle^{N=CH_2}_{COOH} + H_2O$$

Tannage is only possible[18] on the alkaline side of the iso-electric point ($-\log C_H{}^+ = 4.8$) of collagen. It may be taken that the formaldehyde masks the amino groups while freeing the carboxyl groups in the collagen molecule. The formaldehyde tanned collagen is hydrolysable by trypsin, although to a lower degree than the untanned hide powder. This particular combination therefore exerts a fairly considerable retarding effect.

Copper Tanned Hide Powder

Ten gram lots of 100-mesh high powder were drummed for 24 hours in 200cc. of 5 and 2.5 per cent $CuSO_4 5H_2O$ solution, made up in a buffer of $-\log C_H{}^+ = 5.9$, then allowed to stand for 24 hours, filtered, thoroughly washed, dried and powdered. Powdering was not very satisfactory, as the tanned powder dried in hard fibrous lumps, not at all friable. Two other sets, tanned in 1.0 and 0.25 per cent $CuSO_4 5H_2O$ respectively, were discarded, as they gelatinized on drying. Analyses of these powders gave:

Powder tanned in solution $CuSO_4$ per cent	Water %	Hide substance %	Cu %
2.5	9.79	89.46	0.13
5.0 a	9.55	89.48	0.61
5.0 b	7.58	91.21	0.67

Sample b was tanned at a different time and was not so finely powdered as sample a.

Tryptic digestion was carried out as before, the results being given in Table 12.

TABLE 12.

Hide powder tanned in $CuSO_4.5H_2O$ solution	*Per cent hydrolysis* Trypsin	Control
5.0 a %	88.5	19
5.0 b %	79	26
2.5 %	86.5	21
0.0 % Untanned	86	13

a and b were different lots, b being less finely powdered than a.

Copper was present in the residual liquors, but almost entirely in an unionized form. It is remarkable to note that hydrolysis is as great with the treated as with the untreated powder, and in the controls the copper treatment actually seems to activate the hydrolysis.

No work appears to have been done on copper tannage, and it is indeed very doubtful whether this treatment can be spoken of as

a true "tannage." The treated hide powder, when wet, feels like wet raw hide powder, and not like wet tanned hide powder, whether the tannage be vegetable, chrome, quinone, or formaldehyde. It may be supposed that we have merely the formation of a salt between an amphoteric electrolyte, on the alkaline side of its iso-electric point, and a metallic action, in accord with Loeb's work[19]. Representing the collagen, as before, as $R<^{NH_2}_{COOH}$, the combination would be:

$$2R<^{NH_2}_{COOH} + CuSO_4 \rightleftharpoons \left(R<^{NH_2}_{COO} \right\}_2 Cu + H_2SO_4$$

Here it is to be noted that the copper combines with the carboxyl group, leaving the amino group free. The copper tanned hide powder is hydrolysable by trypsin or water to a degree as great as the untanned hide powder. This combination therefore appears to exert no retarding effect whatever on the hydrolysis.

Vegetable Tanned Hide Powder

For purposes of comparison, 10 g. of 100-mesh hide powder was tanned in 200cc. of 5 per cent gallotannin solution at $-\log C_H{}^+ = 5.9$ for 48 hours, then thoroughly washed, dried out at 40°, and powdered. Analysis of the product gave: 5.44 per cent water, 72.23 per cent hide substance, 22.33 per cent tannin (by difference). This was digested with trypsin as before. The tanned hide powder gave 48 per cent hydrolysis with trypsin and 19 per cent in the blank. The residual liquors from the trypsin gave a very faint positive gelatin-salt test for tannin.

There are innumerable theories of vegetable tanning, chemical, physical and colloidal, but until more is known of the chemical nature of tannins and collagen, it would be idle to speculate on exactly what type of combination occurs. Accepting Fischer's view of gallotannin as of the nature of a penta digalloyl glucose, it would seem reasonable to suppose that combination is largely with the basic groups of the collagen molecule. Considering the above experiment in conjunction with those on the other types of tannage, this view would seem to be upheld.

Review of Results on Tanned Hide Powder

In the preceding experiments two instances were found where tannage is believed to consist in combination with the amino group (e. g., with quinone and formaldehyde), and one instance where tannage probably consists in combination with the carboxyl group (e. g., with copper sulphate), where the tanned collagen was hydrolysable by trypsin. In yet another instance, that of chrome tannage, which is sometimes believed to consist in a union of chromium with the carboxyl group, the product was not hydrolysable by trypsin. In view of the first three instances cited, it would follow that chrome

tannage must consist of some other kind of combination, and is obviously a more complicated process in its nature.

It appears also that where combination of the tanning agent occurs with the carboxyl group of the collagen, both tryptic and ordinary hydrolysis are as great as with untanned hide powder, such being the case with the copper tannage. Where combination of the tanning agent occurs with the amino group, it appears to depend on the nature of the linkage what degree of hydrolysis will be obtained. Naturally the greater the amount of tanning agent combined, the less the hydrolysis.

The results of the action of trypsin on tanned collagen are of a preliminary nature only, and suggest a fertile field for further investigation on the mode of action of trypsin and possibly also on the nature of tanning. The possibility of combinations of the tanning agent with some of the polypeptide (—NH. CO. —) linkage has not been considered, as the theory of tannage has not yet reached the stage where this would be profitable. Nevertheless, it is a possibility to be borne in mind in future work, particularly as it is at these linkages where hydrolysis probably occurs.

BIBLIOGRAPHY

[1] G. Mann, "The Chemistry of the Proteids," 556-7 (1906) Macmillan & Co., Ltd., London; The Macmillan Co., New York.

[2] R. A. Seymour-Jones, *Collegium* (London), 1, 296 (1915).

G. Rosenthal, Dissertation (Columbia), 1916.
J. Am. Leather Chem. Assoc., 11, 467 (1916).
J. A. Wilson, *J. Am. Leather Chem. Assoc.*, 12, 108 (1917).
J. T. Wood, *Applied Chemistry Reports*, 2, 368 (1917); Society of Chemical Industry, London.
H. R. Procter, "Principles of Leather Manufacture," 2nd Edn., 138 (1922); E. & F. N. Spon, Ltd., London.

[3] R. H. A. Plimmer, "The Chemical Constitution of the Proteins," 2nd Edn., Part II, 11 (1913); Longmans, Green & Co., London and New York.

[4] W. Kühne, *Verh. d. Naturhist. Med. Ver. in Heidelberg*, 1 (N.S.), 198 (1887).

[5] A. Ewald and W. Kühne, *Ibid.*, 1 (N. S.), 451 (1887).

[6] A. Ewald, *Z.f.Biol.*, 26 (N.S. 8), 1 (1890).

[7] W. M. Bayliss, "The Nature of Enzyme Action," 4th Edn., 80 (1919); Longmans, Green & Co., London and New York.

[8] A. C. Andersen, *Biochem. Z.*, 70, 344 (1915).

[9] E. Abderhalden and P. Rona, *Z. Physiol. Chem.*, 67, 411 (1910).

[10] J. T. Wood, "The Puering, Bating, and Drenching of Skins," 65, 71 (1912); E. & F. N. Spon, Ltd., London.

[11] G. Rosenthal, *loc. cit.*

[12] J. A. Wilson and A. F. Gallun, *J. Ind. Eng. Chem.*, 14, 834 (1922).

[13] L. Michaelis and H. Davidsohn, *Biochem. Z.*, 30, 481 (1911).
Cf. also C. A. Pekelharing and W. E. Ringer, *Z. physiol. Chem.*, 75, 282 (1911); L. Michaelis and H. Davidsohn, *ibid.*, 76, 385 (1912).

[14] L. Meunier, *Chim. et Ind.*, 1, 71, 272, (1918).
F. C. Thompson and W. R. Atkin, *J. Soc. Leather Trades' Chem.*, 6, 207 (1922).
F. L. Seymour-Jones, *J. Ind. Eng. Chem.*, 14, 832 (1922); *Ind. Eng. Chem.*, 15, 265 (1923).

[15] L. Meunier, *Collegium*, 1908, 195. *Monit. Scient.*, (4) 23, 91 (1909).

[16] L. Meunier, *Chim. et Ind.*, 1, 71, 272 (1918).

[17] W. Fahrion, *Monit. Scient.*, (4) 25, 351 (1911); (4) 28, 112, (1914).

[18] A. M. Hey, *J. Soc. Leather Trades' Chem.*, 6, 131 (1922).

[19] J. Loeb, "Proteins and the Theory of Colloidal Behavior," (1922), McGraw-Hill Book Co., Inc., New York and London.

VITA

Frank Leslie Seymour-Jones was born at Wrexham, North Wales, on November 9, 1896. He was educated at Wellington (now Wrekin) College, Wellington, Salop, England, from 1907 to 1913, and matriculated at the University of Leeds, Leeds, England, in September, 1913. In November, 1914, he left to join the British Army, and returned to the University in February, 1919. He graduated thence in July, 1921, as B.Sc., with first class honors in the School of Applied Chemistry of Leather Manufacture, being granted an M.Sc. *in absentia* in July, 1922. Since September, 1921, he has been engaged in graduate study and research in the Department of Chemistry of Columbia University. While at Leeds he held a Brown Scholarship and on graduation was awarded the Le Blanc medal for applied chemistry. He held a Goldschmidt Fellowship of Columbia University, 1921-2, and an 1851 Exhibition Science Research Scholarship (on the nomination of the University of Leeds), 1921-3. He graduated A. M. Columbia in October, 1922. In 1918 he was awarded a Military Cross for "services during the operations culminating in the capture of Jerusalem."

He has published the following scientific papers:

Early Advances in Leather Chemistry
J. Soc. Leather Trades' Chem., 4, 119 (1920).

The Use of the Microscope in Leather Manufacture (with A. Seymour-Jones)
Ibid., 5, 56 (1921).

The Chemical Constituents of Skin
J. Ind. Eng. Chem., 14, 130 (1922).

Mineral Tannages
Ibid., 14, 832 (1922).

The Lime Water Method for the Acidity of Tan Liquors
J. Soc. Leather Trades' Chem., 6, 399 (1922).

Note on the Formation of Gelatin from Hide Powder
Ibid., 7, 93 (1923).

The Colloid Chemistry of Basic Chromic Solutions
Ind. Eng. Chem., 15, 75 (1923).

The Electrophoresis of Chromic Solutions
Ibid., 15, 265 (1923).

Art and Science of Leather Manufacture
Chem. Met. Eng., 27, 1110, 1253 (1922); 28, 200, 400 (1923).